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The Effectiveness of Ultrasound Treatment on the Germination Stimulation of Barley Seed and its Alpha-Amylase Activity

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Abstract**—**In the present study, the effects of ultrasound as emerging technology were investigated on germination stimulation, amount of alpha-amylase activity on dry barley seeds before steeping stage of malting process. All experiments were carried out at 20 KHz on the ultrasonic generator in 3 different ultrasonic intensities (20, 60 and 100% setting from total power of device) and time (5, 10 and 15 min) at constant temperature ($30\,C^{\circ}$). For determining the effects of these parameters on enzyme the Fuwa method assay based on the decreased staining value of blue starch–iodine complexes employed for measurement an activity. The results of these assays were analyzed by Qualitek4 software using the Taguchi statistical method to evaluate the factor's effects on enzyme activity. It has been found that when malting barley is irradiated with an ultrasonic power, a stimulating effect occurs as to the enzyme activity.

Keywords**—**ultrasound, alpha-amylase activity, stimulation and Taguchi statistical method.

I. Introduction

AMYLASES (E.C: 3.2.1.0) are a class of hydrolases widely distributed in nature, i.e. in the higher plants, animals, and microbes. They can specifically cleave the O-glycosidic bonds in starch, a principal storage polysaccharide present in seeds of various plants and other related oligo-and polysaccharides. These enzymes have a great significance with extensive biotechnological applications in food, brew, textile, and paper industries. Alpha-amylase, one of the most valuable enzymes is important in the metabolism of maltose and maltodextrins. Starch depolymerization by amylases is the basis for several industrial processes such as the preparation of glucose syrups and brewing. Cereal alpha-amylases play a very important role in the starch metabolism in developing as well as germinating cereals [1].Industrial applications generally require amylases with a high activity profile. For this purpose much effort to increase the alpha-amylase activity in the germination process of barley has been taken. Various articles have reported the increase of alpha-amylase activity in biologic, physiology and biochemistry literature. All of these were based on the endosperm modification and alourn protoplast to create suitable condition to do anabolic reactions in amylase synthesis sites to increase the amylolytic enzymes activity [2] or the use of natural / artificial chemicals such as gebberllin [3] / ethylene [4] for treatment of barley in order the release of amylase from barley alourn layer. Unfortunately the chemicals methods have the disadvantages that malt contains residues due to the treatment. In order to avoid these unfavorable results developing the physical methods to increase the germination rate and the alpha-amylase activity are indispensable.

Reports and the intriguing possibility that the use of ultrasound may enhance the stimulation of some seeds have led us to examine the feasibility of ultrasound-induced favorable germination of barley seeds in order to increase it's alpha-amylase activity. In recent years, extensive efforts have applied sonication under dry conditions witch may be carried out up to several months before actual sowing. Examples of the use of this process include ultrasonic treatment leading to a three fold enhancement in sunflower seed germination in soil and a ten-day reduction in the ripening time of tomatoes or shorter period to form a gel as a result in a faster release of starch during subsequent cooking in a sonicated rice grain in water. Detailed discussions on the results of these investigations can be found in various reviews and books [5],[6].

In biotechnological processes, ultrasonication method is widely used for laboratory scale and it does not require sophisticated equipment or extensive technical training. The structure and function of biological molecules can be changed by the ultrasound irradiation. The most common interaction mechanisms which involved in this case are either heat or chemical effects and acoustically induced cavitational activity. In addition to these, acceleration the rate of influx or uptaking of a substance into a seed by ultrasonication can also be caused by mechanical effects, i.e. shear stress developed by eddies arising from shock waves. Despite the fact that sonication have applied treating the extensive range of the seed types[7-11] as yet, based our knowledge there is a no scientific literature about the application of ultrasonic waves for stimulating the germination of barley seed and the level of

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it's alpha-amylase activity. The objective of this study is the investigation of the ultrasound effects to treat barley seeds before steeping for grater and faster germination as well as the influence of quicker germination on the alpha-amylase activity. For this purpose the day of germination and alpha-amylase activity were kept as a means of determining rate and yield of process respectively.

II. MATERIALS AND METHOD

A. Materials

Chemicals

All chemicals with high analytical grade including iodine, KI (potassium iodide), KH_2PO_4 (monobasic phosphate), $K_2HPO_4{\cdot}3H_2O$ (dibasic phosphate) and soluble starch from potato (S-2630) which used for alpha-amylase assay were obtained from Sigma-Aldrich, Fluka and Merk companies.

Raw Materials

Karon in kavir barley varieties with moisture content of 9% and an average content of protein 11.5% was used in all experiments. To prevent absorption of moisture it was stored in a dry place at $20\,C^\circ$ until malting. Also it must be mentioned, that for removal of dormancy, samples were stored at room temperature (25–37 C°) for 3 months after harvest.

Equipments

The sets of Gerhardt Kjeldatherm and Gerhardt Vapodest 30 instrument were used for determining the amount of protein in barley seeds.

Ultrasonic irradiation was given by means of ultrasonic generator UP 200 H horn type (20 kHz, maximum wave amplitude of 210 μm and maximum nominal power output 460W) equipped with a radial Sonotrode S3 (3mm diameter) designed by Dr. Hielscher GmbH (Treptow, Germany).

B. Experiments Design

In this study 4 important effective parameters namely, ultrasound intensity, the time of ultrasonic irradiation, temperature and frequency were selected. Since in our design problem the operating temperature and frequency were fixed, only 2 variables were remained for the design of experiments. Taguchi method at our disposition was employed to design experiments condition and evaluate the factor's effect on stimulation and activity of enzyme. L9 orthogonal array is used to design of experiments. 9 experiments, repeated for 3 times, considering the 2 parameters as defined above in the 20 KHz frequency were done for barley seeds. Qualitek4 analysis using the ANOVA approach was employed for finding the average effects of individual parameters on enzymatic process condition as shown in the figures2 and 3 according to software output.

C. Experimental

Sonication of sample

The ultrasonication experiments were carried out at 20 kHz on the ultrasonic generator. The tip of the horn was immersed about 9mm into the solution to be processed. all experiments were performed on samples (10 g barley seeds) dispersed in 80 ml of tap water in direct sonication at ultrasonic intensity of 20, 60 and 100% power setting of device with additional agitation or shaking that was employed, to avoid standing waves or the formation of solid free regions for the uniform distribution of the ultrasonic waves. The ultrasonic energy was pulsed using a duty cycle control in order to reduce the formation of free radicals. The cycle was set on 50% in all experiments. The solution was processed at constant temperature of $30\,C^\circ$ with the sonication horn for 5, 10 and 15 min. The temperature inside the solutions was intermittently checked.

Malting Stage

Barley seeds were micromalted manually in laboratory scale according to the following procedure: samples after stepping at 16-17 C° for 6 h in the incubator chamber, were air-rested for 8h .This process was done 3 times periodically to reach a moisture content of 45% and the subsequent germination phase followed 96 h with keeping the 45% moisture content (with watering the samples every four hours).Then the samples were kilned in the drying oven in gradually ramping temperature from 17 to $55\,C^\circ$ over 20 h, from 55 to $65\,C^\circ$ over 20 h, from 65 to $75\,C^\circ$ over 6 h and finally from 75 to $82\,C^\circ$ over 4 h . The drying process was stopped with reaching the moisture content of samples to 4%. Afterwards with removing the rootlets, the samples were milled and the malted flour was prepared for the next stages of the experiments [12].

Extraction of Enzymes from Malt

In this research commonly 50 mM Na-phosphate buffer with pH=8 was used as the best extraction media. This buffer enhances the release of more enzyme rather than another media such as the mixture of NaCl in water owing to the fact that either the high pH or added phosphate ions (higher concentration), as pointed out by Osman[12]. Approximately, 0.75 g malt flour was weighed in duplicate into centrifuge tubes and 4 mL extraction media was added with mixing. Extraction was performed for 30 min at $30\,C^\circ$ with regular vortexing for 5 s at 5 min intervals and was terminated by centrifugation for 10 min at 2826*g* [12].

Determination of Alpha-Amylase Activity Based on Decrease in Starch/Iodine Colour Intensity

Starch forms a deep blue complex with iodine and with progressive hydrolysis of the starch, it changes to red brown. Several procedures have been described for the quantitative determination of amylase based on this property. This method determines the dextrinising activity of alpha-amylase in terms of decrease in the iodine colour reaction. The dextrinising

activity of alpha-amylases employs soluble starch as substrate and after terminating the reaction with dilute HCl, iodine solution is added. The decrease in absorbance at 620 nm is then measured against a substrate control. One percent decline in absorbance is considered as one unit of enzyme [13].

Enzyme Assay

starch–iodine assay according the Fuwa method was carried out as follows: assay reactions were initiated by adding 0.5mol of starch solution (20 mg/mL in 0.1 M phosphate buffer pH=7) and 0.5mol of enzyme in 0.1M phosphate buffer at pH 8.0 to reaction tube and incubated at 37°C for 30min. The reaction is then terminated by adding 1ml of 1N HCL. Following reaction termination, the mixture then diluted to nearly 10 ml with H2O, followed by the addition of 1ml of iodine reagent (0.2% iodine and 2% potassium iodide). Finally, the volume is adjusted to 10ml with distillated water and the amount of color development is determined by measuring the absorbance at 620 nm [14].

III. Result and Discusion

The efficacy of ultrasonication on barley stimulation was investigated at $30\,C^{\circ}$ and cavitation levels between 20 and 100% of output power of device. Resulting absorbance are showed in figure1. Concerning the sonication process the following results have been obtained: by using the barley that treated above, the germination period is shortened to 4 to 5 days depending upon the ultrasonic intensity and exposure time to ultrasonic waves, from the usual 7 days and the absorbance (corresponding with alpha-amylase activity) increased from 1.55 for 0% power setting to 1.639 for a power setting of 100% at the end of the 15 min processing time (fig.1). This may be mostly due to acoustic cavitation, which consists of the formation, growth and violent collapse of small bubbles or voids in liquid [15].

Cavitation may also result in physiological or biochemical changes in the seed which prime the germination process so that upon exposure of the seed to germination conditions, less time is needed for seed to initiate germination. One mechanism proposed for causing physiological or biochemical changes ascribed to the shell fragmentation by cavitation. Shell fragmentation due to the mechanical effects of ultrasound (shock waves and microjets) dramatically facilitates the passage of the water molecules across the cell wall and induced the grater amount of alpha-amylase release and caused a speeding up of metabolic processes in living cells. Also ultrasonication produced numerous small holes in the coating and after steeping in the water a significant rise in seedling moisture resulted.

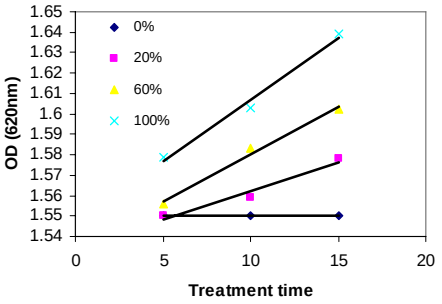

Fig. 1 Alpha- amylase optical density vs. time, barley sonication before steeping at $30\,C^{\circ}$ ($OD=OD_{control}-OD_{sample}$)

Qualtiek4 Statistical Analysis of Barley Ultrasonication before Steeping

Table1 shows the detailed analysis of variance results of experiments conducted under Taguchi method. In this table the contribution of each factor quantitatively was determined by using ANOVA approach. Also figures 2 ,3 depict the main effects of ultrasonic power and exposure time respectively. By the term "main effects", the average of obtained results (as an optical density), in which each factor is at a given level, is meant. As it is shown in these figures the average effects of ultrasound intensity and the time of exposure to ultrasound were positive on enzyme activity. Also the findings indicated that both treatment time and cavitation level with a contribution percentage as high as 47.015% and 46.588% respectively, had the dominant effects on overall performance.

It must be mentioned, at these curves longitudinal axis is a levels of selected parameters such as p= ultrasonic intensity and t= the time of ultrasonic irradiation and transverse axis is an amount of absorbance as the criteria of the process yield in statistical analysis.

TABLE 1
ANALYSIS OF VARIANCE (ANOVA)

No.	Factors	DOF	Sums of Squares	Variance	F-Ratio	Pure Sum	Percent
1	P	2	0.008	0.004	95.677	0.008	46.588
2	t	2	0.009	0.004	96.545	0.008	47.015
Other/Error		22	0.000	0.000			6.397
Total		26	0.018				100.000%

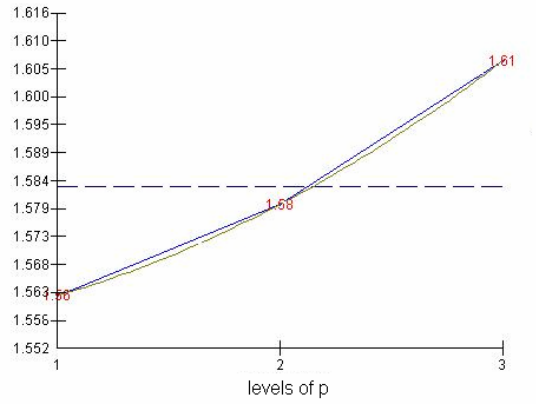

Fig. 2 Average effect of ultrasound intensity by Taguchi method using qualitek4 software

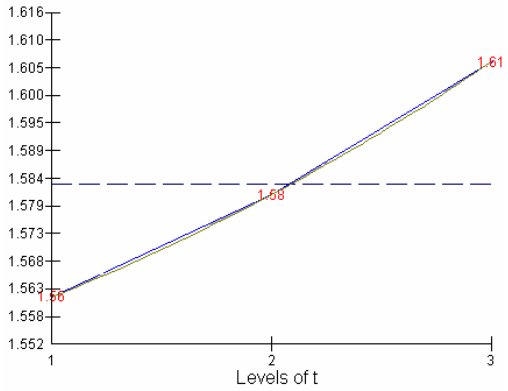

Fig. 3 Average effect of time by Taguchi method using qualitek4 software

IV. CONCULTION

For the first time ultrasonication is provided a modified malting process (without additive) which the level of alpha-amylase released was higher than the alpha-amylase levels found in malt produced under conventional malting methods. Our opinion for this fact is that the ultrasonic treatment of dried barley seeds in aqueous media results in an advanced hydration process with concurrent shell fragmentation. This is common explanation for exhibition enhanced germination by a reduction in the time required for germination in the sonicated seeds, and an increase in the barley's alpha-amylase activity.

The action of ultrasound is quite effective in stimulation the germination of barley suggesting that this technique has interesting possibilities in horticulture and brew industry. Treated seeds by this method can be dried, stored, and germinated at a later date while maintaining its accelerated germination characteristics.

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